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Mitochondrial Glycerol Kinase Activity in Rat Brain

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Glycerol kinase activity was found in the particulate fraction of rat brain homogenates predominantly associated with mitochondria. The enzyme remained bound to the particulate fraction after treatment with a variety of solubilizing agents.

The oxidation of glycerol to CO_2 has been demonstrated by using both slices (Sloviter *et al.*, 1966; Sloviter & Suhara, 1967) and homogenates of brain (Tildon *et al.*, 1975), but the enzymic machinery for this process has not been described.

Toews (1967) has suggested that glycerol oxidation proceeds through an initial dehydrogenation step to glyceraldehyde, but it seems unlikely that glycerol dehydrogenase could contribute significantly to this oxidation, since that enzyme has a high K_m (0.1 M) for glycerol. It seems more plausible that glycerol oxidation proceeds via the terminal portion of the Embden–Meyerhof pathway. Both glycerol 3-phosphate dehydrogenase (NAD^+) (EC 1.1.1.8) and glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) have been demonstrated in this tissue (Garcia-Bunuel *et al.*, 1962; Hemon, 1968; Burch *et al.*, 1974), but the presence of glycerol kinase (EC 2.7.1.30) has been questioned. One report has indicated a low glycerol kinase activity in brain (Goodner *et al.*, 1973), but other studies have concluded that the enzyme was essentially absent (Wieland & Suyter, 1957; Vernon & Walker, 1970). Since the proposal of other pathways seemed speculative and without firm experimental support, we have re-examined the brain for glycerol kinase activity. The present paper describes a glycerol kinase activity in brain that is predominantly associated with the mitochondria. The enzymic activity is more than sufficient to account for the rate of glycerol oxidation *in vitro* by the brain.

Experimental

Albino Wistar rats (2–5 months old), weighing 150–300g and maintained on Purina Laboratory Chow, were used. The whole brain was removed after decapitation and placed in a Petri dish at 4°C. The brain was homogenized in 0.01% KCl in 1.0mM-EDTA by using a TenBroeck homogenizer with a clearance of 0.25–0.04mm. The homogenate was centrifuged at 250*g* for 10min to remove nuclei and cellular debris; the supernatant was then spun for an additional 15min at 17000*g* in a Sorval centrifuge. The pellet was washed and then resuspended in the EDTA/KCl solution to an approximate protein concentration of 10mg/ml (usually one-tenth of the original volume of homogenate). This 17000*g* particulate suspension was used in most assays; however, in several experiments, whole homogenates, 17000*g* supernatant, as well as myelin, synaptosomes and mitochondria were used. These latter subcellular fractions were obtained by the method of Gray & Whittaker (1962).

Reagents and chemicals

ATP, ADP, AMP, NAD^+, fructose 1,6-diphosphate, glucose 6-phosphate, Triton X-100, α-glycerol phosphate and enzymes were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; Lubrol-WX was given by ICI America, Stamford, CT, U.S.A. Other chemicals were certified reagents from Fisher Chemical Co., Pittsburgh, PA, U.S.A.

Enzyme assay

Glycerol kinase was assayed fluorimetrically by using a modification of the method of Wieland (1957). This assay takes advantage of the sequential enzymic reactions:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{glycerol 3-phosphate} + \text{ADP}$$

$$\text{Glycerol 3-phosphate} + \text{NAD}^+ \xrightarrow{\text{Glycerol phosphate dehydrogenase}} \text{dihydroxyacetone phosphate} + \text{NADH} + \text{H}^+$$

in which glycerol is first converted into glycerol 3-phosphate and then in the presence of excess of

glycerol phosphate dehydrogenase is stoicheiometrically oxidized to dihydroxyacetone phosphate with hydrazine as the trapping agent. Unless otherwise indicated, the reaction mixture usually contained 0.9ml of buffer (0.6M-glycine, 6mM-$MgCl_2$ and 3M-hydrazine, pH9.8), 0.05ml of 0.09M-ATP, 0.07ml of 0.02M-NAD^+, 0.1ml of 0.1M-glycerol, 0.03ml of glycerol phosphate dehydrogenase (1450 units/ml; 1 unit = 1.0μmol of substrate transformed at pH7.4 at 25°C per min), the enzyme preparation (0.02–0.1ml) and water, in a final volume of 3ml. The activity of the enzyme was calculated from the production of NADH, as determined by the linear increase in fluorescence, by using an Aminco–Bowman spectrophotofluorimeter with an excitation wavelength of 340nm and an emission wavelength of 466nm. All assays were carried out at 25°C and two blanks were included with each determination, one omitting ATP and the second omitting glycerol. The assay was sensitive enough to detect the production of 0.03nmol of glycerol 3-phosphate/min. Measurements for glycerol kinase were carried out by using whole brain homogenates, the 17000*g* precipitate and the supernatant as well as the myelin, mitochondrial and synaptosomal fractions. Since glycerol was present in the glycerol phosphate dehydrogenase solution, the glycerol content of the incubation mixture was measured for more accurate K_m determinations. Glycerol was measured by using a modification of the enzyme assay, in which purified glycerol kinase from *Candida mycoderma* was added to the reaction mixture. The reaction was allowed to go to completion and the concentration of glycerol was calculated from the increase in fluorescence. The recovery of added glycerol was $\geq 95\%$ in ranges from 0.5 to 20μmol. In several experiments, glycerol kinase activity was measured by the method described by Burch *et al.* (1974). The enzyme activity measured by using this method was essentially the same as that obtained by using the assay described above. Protein concentrations were determined by the method of Lowry *et al.* (1951) as described by Layne (1957).

Results

Significant amounts of glycerol kinase activity were found in the 17000*g* particulate fraction of rat brain homogenates, usually about 0.7–1.1nmol/min per mg of protein. The activity was proportional with time for up to 20min and proportional to protein concentrations, as indicated by a typical experiment in which the rate of glycerol 3-phosphate production was 0.21, 0.49 and 0.69nmol/min respectively when 0.15, 0.37 and 0.73mg of protein from the particulate fraction was added to the reaction mixture. No activity could be detected in the supernatant, and the measurement of activity in the whole homogenate was complicated by a large non-specific increase in fluorescence, which gave high blanks. However, the net change in fluorescence with the homogenate was equivalent to 0.2–0.3nmol/min per mg of protein. When either ATP, glycerol or glycerol phosphate dehydrogenase was omitted from the reaction mixture, or the enzyme was heated at 100°C, the activity was less than 5% of that with the complete system. Measurements of endogenous glycerol and the addition of various amounts of this substrate revealed that the enzyme activity was proportional to glycerol concentration, with an apparent K_m of 2.9×10^{-6}M. Attempts to solubilize the enzyme revealed that it was tightly bound to the particulate fraction. Treatment of the 17000*g* (P_2) fraction with Triton X-100 (0.3mg/mg of protein), Lubrol-WX (0.2%) or sonication (Branson Sonicator setting 5, for times indicated in Table 1) resulted in some soluble enzyme activity, but the bulk of the kinase remained bound to the particulate fraction (Table 1). The combination of Lubrol-WX treatment and sonication resulted in the solubilization of about 50% of the activity, and this should permit the further purification of this enzyme and a more rigorous characterization of its chemical and physical properties. However, treatment with deoxycholate completely destroyed the activity.

Because of the unique subcellular localization of this enzyme, the crude mitochondrial fraction (P_2) was further separated into subcellular fractions to determine if glycerol kinase was more localized in the synaptosomal or mitochondrial fractions. Table 2 shows the specific activity of glycerol kinase in the subcellular fractions of the brain. The specific activity associated with the mitochondrial fraction was fourfold higher than the activity associated with the synaptosomal fraction. In addition, more than 70% of the total activity was recovered from the mitochondrial fraction in two separate experiments.

Discussion

The results described above provide cogent evidence for the presence of glycerol kinase activity in rat brain. In a survey of several different tissues, Vernon & Walker (1970) concluded that there was no significant activity of glycerol kinase in the brain, and much earlier Wieland & Suyter (1957) had come to a similar conclusion. A possible explanation for these conclusions could have been the low sensitivity of the method or the presence of additional enzymes in the tissue extract that could convert the product glycerol 3-phosphate into other intermediates. Goodner *et al.* (1973) have reported the presence of glycerol kinase in brain. However, their results were based on an assay system that gave non-linear rates with untreated tissue. While the present paper was in preparation, Jenkins & Hajra (1976) described a

Table 1. *Glycerol kinase activity after treatment with solubilizing agents*

In these experiments a 10% (w/v) homogenate was prepared from the brains of six rats. After removal of the nuclei and cell debris, the supernatant (S_1) fraction was centrifuged and the 17000*g* precipitate was washed three times and reconstituted in a medium containing 0.025M-sucrose, 0.001M-EDTA and 0.1M-glycerol. The suspension was divided into batches and treated as indicated. After treatment, the samples were spun at 20000*g* for 15min; the precipitate was resuspended, and this and the supernatant were assayed for glycerol kinase activity. The concentrations of Lubrol-WX and Triton X-100 were 0.3mg/mg of protein and 0.2% respectively; the concentration of deoxycholate was 0.3%. The sonication experiments were performed by using a Branson Sonicator. The results with deoxycholate were obtained in an independent experiment, and the amount of protein does not reflect a recovery. The enzyme assay is described in the text.

Treatment of 17000*g* particulate fraction	Activity (nmol/min per mg of protein)	Protein (mg/ml)	Total activity (nmol/min per ml)
None	0.71	17.9	13.75
Triton X-100			
Supernatant	0.29	2.58	0.8
Precipitate	0.70	10.35	7.2
Lubrol-WX			
Supernatant	1.45	2.43	3.5
Precipitate	0.70	11.64	8.1
Sonication (1min)			
Supernatant	0.51	2.68	1.4
Precipitate	0.50	10.67	5.3
Sonication (3min)			
Supernatant	0.91	3.84*	3.5
Precipitate	0.74	14.06	10.4
Sonication (6min)			
Supernatant	0.46	6.05	2.8
Precipitate	0.33	13.87	4.6
Lubrol-WX+sonication (3min)			
Supernatant	0.51	13.00*	6.6
Precipitate	0.84	4.94	4.2
Deoxycholate			
Total fraction	<0.05	—	—

* In these two instances the amount of protein in the supernatant fraction was calculated as the difference between the total and the measured amount of protein remaining with the precipitate.

Table 2. *Glycerol kinase distribution in subcellular fractions of rat brain*

Rat brain homogenates and subcellular fractions were prepared by differential centrifugation by using a discontinuous sucrose gradient of 0.32, 0.8 and 1.2M as described by Gray & Whittaker (1962). The distribution of protein in the various fractions was similar to the values reported by Bradford (1969), and the total glycerol kinase activity recovered in the synaptosomal and mitochondrial fractions was 32 and 88nmol/min respectively. In a second experiment, using half as much of the crude mitochondrial fraction (P_2), the distribution of total activities in synaptosomal and mitochondrial fractions was 17.9 and 43.9nmol/min respectively. Enzymic activity was determined as described in the text.

Fraction	Activity (nmol/min per mg of protein)
Homogenate	0.32
P_1 (nuclear pellet)	0.21
S_2 (17000*g* supernatant)	0.02
P_2 (17000*g* pellet)	0.70
P_2A (myelin)	0.08
P_2B (synaptosomes)	0.4
P_2C (mitochondria)	2.2

glycerol kinase in brain, measured by using a radiochemical assay. The properties of the enzyme were similar to those described above, except that the K_m for glycerol was higher.

The demonstration of glycerol kinase in the brain should lessen speculation about a novel pathway for glycerol oxidation by this tissue (Coxon & Coles, 1975). In fact, the amount of glycerol kinase in the brain can account for the formation of glycerol 3-phosphate at a rate of 0.5–2.0μmol/h per g wet wt. This rate is manyfold higher than the rate of oxidation of glycerol *in vitro* by slices or homogenates (Tildon *et al.*, 1975).

One striking difference between the brain enzyme and that obtained from other tissues is its subcellular location. In the brain, the enzyme is tightly bound to the particulate fraction, whereas in other tissues this enzyme is almost always soluble. Unlike several other particulate enzymes (Wilson, 1972; Hochman *et al.*, 1974), brain glycerol kinase does not manifest any 'latent' activity after the addition of Triton X-100 or other detergents.

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